Immunohistochemical Localization of Membrane and α-Granule Proteins in Plastic-Embedded Mouse Bone Marrow Megakaryocytes and Murine Megakaryocyte Colonies

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Using an immunoperoxidase technique that permits optimal antigen localization at the light microscope level, we have detected two platelet α-granule constituents and three platelet membrane glycoproteins in mouse bone marrow megakaryocytes and in murine megakaryocyte colonies grown in soft agar culture for three to seven days. Using polyclonal antibodies prepared against human platelet proteins, we have demonstrated labeling for von Willebrand factor, fibrinogen, and the membrane glycoproteins IIa and GMP-140 in both bone marrow megakaryocytes and megakaryocyte colonies after seven days of culture. Using monoclonal antibodies to membrane glycoproteins IIb and GMP-140, we have demonstrated label in mouse bone marrow megakaryocytes. Granulocyte and macrophage colonies were negative for each of these markers. Murine bone marrow megakaryocytes and megakaryocyte colonies demonstrated a similar enzyme histochemical pattern: weakly positive for α-naphthyl acetate esterase and negative for chloroacetate esterase. These data indicate that megakaryocytes grown in soft agar culture express many of the same glycoproteins as bone marrow megakaryocytes. Furthermore, the ability of antibodies directed against human platelet membrane glycoproteins to identify murine megakaryocyte glycoproteins indicates that these constituents have been highly conserved during evolution.

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MATERIALS AND METHODS

Animals
C57BL/6J mice, obtained from Simonsen Laboratories, Inc (Gilroy, Calif.), were used for these experiments. These mice were bred under specific pathogen-free conditions and then maintained under conventional conditions. Guinea pigs and rats were purchased from EZH Co (Williams, Calif.).

Preparation of Murine, Guinea Pig, and Rat Bone Marrow Specimens
Mouse femoral marrow samples were obtained from animals killed by cervical dislocation and guinea pig and rat femoral marrow samples were obtained from animals killed by ether anesthesia. The femur was split lengthwise and the fragments of marrow gently pushed out from the bone into 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, at 4 °C. Specimens of marrow were fixed for four to six hours at 4 °C.

Preparation of Murine Colony Samples
Spleen cells were obtained as previously described.14 All cultures were performed in an agar medium (final concentration of agar was 0.3% in Dulbecco’s modified Eagle’s medium).15 Final concentration of horse serum was 20%. One mL of a cell suspension in agar medium was cultured with 0.1 mL of pokeweed mitogen stimulated spleen cell-conditioned medium, and incubated for three to seven days in a humidified atmosphere of 10% CO2 in air. At various times, colonies were either removed from culture dishes with a finely drawn Pasteur pipette and placed in the fixative solution, or fixative was gently poured over the entire agar disc. In the latter case, the discs then were gently removed from the 35 mm plastic dishes and placed in a larger volume of fixative. All samples were fixed at 4 °C in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4, for four to six hours.
Preparation of Bone Marrow and Colony Samples for Plastic Embedding

The following steps were performed at 4 °C: Colonies in suspension were processed by allowing them to settle by gravity after each step. After fixation, samples were rinsed three times with 0.1 mol/L phosphate buffer which contained 2% sucrose and 50 mM NH₄Cl. The samples were then dehydrated in 50% acetone for 0.5 hour, 100% acetone for 0.5 hour, and 50 parts acetone/50 parts glycol methacrylate monomer for 0.5 hour followed by infiltration with 100% glycol methacrylate monomer overnight. Subsequently, the specimens were embedded in a mixture composed of glycol methacrylate monomer (20 mL), benzol peroxide (0.09 g), and polyethylene glycol 400 with N,N-dimethylaniline (0.5 mL; Polysciences Inc, Warrington, Pa). The embedded tissue was placed under vacuum (15 to 20 mm Hg) at 4 °C and the plastic allowed to polymerize overnight. One to three um sections were then cut with glass knives on a Sorvall JB-4 microtome. The sections were transferred via water to coverslips and air dried at room temperature.

Enzyme Histochemical and Immunohistochemical Procedures

Routine staining procedures and enzyme histochemistry were performed as previously described.²³ The immunohistochemical procedure²⁷ is a modification of a biotin-avidin procedure. Immunohistochemical procedures on plastic sections were performed in the following sequence. Unless otherwise specified, all incubations were carried out at room temperature.

(a) Digestion with 0.25% trypsin in Saline A (NaCl, 150 mmol; KCl, 5 mmol; glucose, 5 mmol; phenol red, 0.01 mmol; NaHCO₃, 7 mmol) (Gibco, Santa Clara, Calif), pH 7.6, for ten minutes at 37 °C.

(b) Incubation with 3% normal goat or horse serum (same species as secondary antibody) in Dulbecco’s Ca+ and Mg+ free phosphate-buffered saline (CMF-PBS) (KCl, 3 mmol; KH₂PO₄, 1 mmol; NaCl, 150 mmol; Na₂HPO₄·7H₂O, 8 mmol; pH 7.36 to 7.45) for 30 minutes at 37 °C.

(c) Incubation overnight at 4 °C with one of the antisera listed below. All dilutions were made in CMF-PBS with 3% normal goat or horse serum (same species as secondary antibody, vide infra) and sections were rinsed between incubations with CMF-PBS.

(1) Polyclonal antibodies: rabbit antihuman von Willebrand factor (Factor VIII–related antigen) (Dako, Santa Barbara, Calif, 1:1000); rabbit antihuman fibrinogen antiserum (Cappel, Malvern, Pa, 1:1000); rabbit antihuman platelet membrane glycoprotein IIIa (I:2000); rabbit antihuman 0-granule membrane glycoprotein (GMP-140) (I:2000); rabbit antismouse platelet antiserum (I:500); rabbit antirat brain acetylcholinesterase (Dr Jean Massoule, Paris, France, 1:100); rabbit antihuman lysozyme (Dako, Santa Barbara, Calif, 1:500).

(2) Monoclonal antibodies: mouse antihuman platelet GMP-140 (S12) (I:500); mouse antihuman glycoprotein IIb (Tab) (I:1500).

(d) Incubation with the appropriate secondary antibody, ie, biotinylated goat antirabbit IgG (Vector, Burlingame, Calif, 1:200) or biotinylated horse antiserum (Vector, 1:200) for one hour.

(e) Inhibition of endogenous peroxidase by incubation with 0.3% H₂O₂ in 30% methanol for three minutes.

(f) Incubation with the avidin-biotin-peroxidase complex (Vector, 1:80) for one hour.

(g) Development of peroxidase reaction by preincubation with 0.05% 3,3-diaminobenzidine in CMF-PBS for ten minutes, followed by incubation with a CMF-PBS solution containing 0.05% diaminobenzidine, 0.1% H₂O₂, 0.1 mol/L imidazole, and 0.1 mol/L sodium azide for five minutes. After washing 3 times in water, the reaction was further enhanced by incubation with a solution of 0.5% CuSO₄ for five minutes.

(a) Counterstaining with Gill’s hematoxylin 2 for 1.5 minutes; bluing with Scott’s water (0.2% Na₂CO₃, 1% MgSO₄ in tap water); and finally drying and mounting with Permount.

Control procedures included the substitution of irrelevant antibodies, preimmune serum, or ascites fluid for the primary antibodies and the elimination of primary and secondary reagents.

RESULTS

We have utilized polyclonal and monoclonal antibodies directed against a variety of human platelet proteins to localize these substances within the megakaryocytes of intact specimens of rodent bone marrow and murine megakaryocytes obtained from soft agar cultures. These proteins can be classified, by their fine-structural localization in platelets, into three groups: (1) plasma membrane proteins, (2) o-granule membrane proteins, and (3) o-granule matrix proteins. Since these antibodies against human platelet proteins strongly cross-reacted with rodent antigens, we were able to investigate their localization in bone marrow cells and in cells at early culture times. Control preparations showed no evidence of nonspecific staining.

Staining With Antibodies to Platelet Plasma Membrane Proteins

Glycoprotein IIIa. Polyclonal antisera against human glycoprotein IIIa labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (Fig 1). Staining in megakaryocytes was distributed throughout the cytoplasm but was most intense on the plasma membrane. No staining of other hematopoietic cells was observed. Guinea pig and rat megakaryocytes from bone marrow specimens were also labeled (not shown), but not as strongly as murine megakaryocytes.

Murine megakaryocyte colonies, fixed and processed three days after initiation of soft agar cultures, were strongly reactive for GP IIIa (Fig 2), as were colonies processed seven days after culture (Fig 3). Granulocyte colonies (Fig 4) and macrophage colonies (Fig 4, inset) from the same preparations were completely unreactive for GP IIIa.

Glycoprotein IIb. Monoclonal antibodies against human glycoprotein IIb (Tab) labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). The staining pattern was similar to that obtained with antibodies to GP IIIa (vide supra).

Platelet Antiserum. Heterologous antiserum produced in rabbits against mouse platelets (PAS) labeled virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). The staining pattern was similar to that obtained with antibodies to GP IIIa (vide supra).

Murine megakaryocyte colonies, fixed and processed five to seven days after initiation of soft agar cultures, were strongly labeled with PAS (not shown). Granulocyte and macrophage colonies from the same preparation were unlabeled with PAS (not shown).
Staining with Antibodies to Platelet α-Granule Membrane Protein 140

Both polyclonal (not shown) and monoclonal (S12) (Fig 5, inset) antibodies developed against the human platelet α-granule membrane protein with a molecular weight of 140 kD (GMP-140) produced labeling of virtually all morphologically identifiable murine bone marrow megakaryocytes. The staining pattern was similar to that observed with the antibodies against platelet α-granule matrix proteins (vide infra) and is consistent with the fine-structural localization of GMP-140 in human platelets.19 Labeling with the monoclonal antibody S12 was weaker than the labeling obtained with the polyclonal antibodies. All other hematopoietic cells were negative.

Polyclonal antibodies produced variable labeling of megakaryocyte colonies processed seven days after initiation of cultures of splenic cells (Fig 5). Some cells exhibited a strong granular label while others, usually smaller in size, appeared negative. The monoclonal antibody S12 produced only weak labeling of megakaryocytes from the same preparation (not shown). Granulocyte and macrophage colonies were negative (not shown).

Staining with Antibodies to Platelet α-Granule Matrix Proteins

Von Willebrand Factor. Antibodies against human von Willebrand factor (VWF) produced strong granular staining in virtually all morphologically identifiable murine bone marrow megakaryocytes (Fig 6, inset A). The distribution was similar to that observed with antibodies to GMP-140. There was no labeling of other hematopoietic cells, but moderate endothelial cell staining was observed (not shown).

Murine megakaryocyte colonies, processed three days (Fig 6, inset B) to seven days (Fig 6) after initiation of cultures, were strongly reactive with antibodies to VWF. Granulocyte and macrophage colonies were negative.

Fibrinogen. Antibodies against human fibrinogen produced a strong granular label in virtually all morphologically identifiable murine bone marrow megakaryocytes (not shown). No other hematopoietic cells were labeled.

Murine megakaryocyte colonies processed five to seven days after initiation of cultures were strongly reactive for fibrinogen (not shown). Granulocyte and macrophage colonies were negative.

Controls

When preimmune rabbit serum was substituted for specific primary polyclonal antiserum, no staining was observed in any cells. Similarly, when either primary or secondary reagents were eliminated, no staining was observed. When ascites fluid was substituted for specific monoclonal antibodies, no staining was observed in any cells.

Other Markers

Polyclonal antisera produced in rabbits against rat brain acetylcholinesterase labeled many, but not all, morphologically identifiable murine bone marrow megakaryocytes (not shown). Polyclonal antisera produced in rabbits against human lysozyme labeled most murine bone marrow granulocytes and macrophages and cells in granulocyte and macrophage colonies, but not bone marrow megakaryocytes or megakaryocytes grown in soft agar culture (not shown).

Both murine bone marrow megakaryocytes and megakaryocyte colonies obtained from seven-day cultures of splenic cells were positive for the enzyme α-naphthyl acetate hydrolysis.
cyes (not shown). Some variability in the degree of staining was noted within the same bone marrow specimen. Granulocyte and macrophage colonies were negative. Murine bone marrow megakaryocytes and macrophages and megakaryocyte and macrophage colonies obtained after seven days of culture were negative for the enzyme chloroacetate esterase (not shown). In contrast, neutrophils in both bone marrow and granulocyte colony samples were strongly reactive for this enzyme (not shown).

**DISCUSSION**

Utilizing monoclonal and polyclonal antihuman antibodies and an immunoperoxidase technique, we have demonstrated the presence of a variety of platelet membrane and alpha granule glycoproteins in murine bone marrow megakaryocytes and in murine megakaryocytes obtained from colonies grown in soft agar cultures. In an earlier report we demonstrated the presence of these glycoproteins in human bone marrow megakaryocytes using the same antibodies and immunohistochemical technique. All antihuman megakaryocyte/platelet antibodies strongly react with murine megakaryocytes. In contrast, antihuman lysozyme antibodies labeled murine granulocytes and macrophages, but not megakaryocytes. Guinea pig and rat bone marrow megakaryocytes were also reactive with antibodies to glycoprotein IIIa (the only antibody tested against these species). Therefore, we have concluded that these glycoproteins are highly conserved. Support for this conclusion has come from a recent study by Kunicki and Newman in which they demonstrated that human platelet membrane glycoproteins IIb and IIIa are structurally conserved in chicken peripheral blood thrombocytes. They showed this by indirect immunofluorescence, using polyclonal and monoclonal antibodies, and two-dimensional polyacrylamide gel electrophoresis, which revealed the incorporation of 35S methionine by chicken thrombocytes into glycoprotein IIb and IIIa analogs in vitro. Unexpectedly, in our study, mouse bone marrow megakaryocytes also labeled with monoclonal antibodies to human platelet membrane glycoproteins, although not as strongly as with polyclonal antibodies. Since the monoclonal antibodies were produced in Balb C mice and were used to examine cells from C57BL/J6 mice, this suggests that there may be some differences between platelet glycoproteins of these species.

At least two types of murine megakaryocyte colonies have been recognized in soft agar cultures. One type is typically composed of 10 to 30 large megakaryocytes which are mature in appearance ("big cell" type). The other ("heterogeneous" type) is composed of more cells (often greater than 100), some of which are small and immature as evaluated in part by staining for acetylcholinesterase, a specific marker for murine megakaryocytes. In our current studies, we observed an increase in average cell size from 3 to 7 days in culture, and the size of megakaryocytes in seven-day colonies (at which time murine megakaryocyte colonies have reached their maximal maturation in our culture system) approximated the size of mature bone marrow megakaryocytes. However, no evidence of platelet production was observed.

Since both colony types appear simultaneously in agar cultures, they probably represent the progeny of different populations of precursors. Our data demonstrate that although each colony type probably originates from different populations of precursors, the appearance of markers, as detected by light microscope level immunohistochemical analysis, is similar in both, since after seven days of culture, the majority of cells in both "big cell" and "heterogeneous" colonies labeled for all proteins tested. We also demonstrated that murine megakaryocyte colonies, present after only three days of soft agar cultures of murine colony-forming cells, labeled for both platelet membrane glycoprotein IIIa and von Willebrand factor, an alpha granule matrix protein. This is significant since cells at this time in culture are morphologically immature; in fact, this is the first day of culture in which murine megakaryocyte colonies may be reliably identified, based upon the relatively large size and refractility of the cells.

Previously, staining for acetylcholinesterase has been used to identify immature megakaryocytes in the mouse. Megakaryoblasts have been identified in human bone marrow by platelet peroxidase, the earliest marker of megakaryocytes, and by antiserum against platelet membrane preparations and platelet membrane glycoproteins. Significantly, Vainchenker et al7 identified human megakaryocytes in plasma clot cultures by the presence of platelet peroxidase, and Mazur et al3 identified human megakaryocyte colonies with immunofluorescence techniques using an antiplatelet glycoprotein antiserum. Significantly, Vainchenker et al and Vinci et al, using monoclonal antibodies, determined that the glycoprotein IIb/IIIa complex is an early antigenic marker of megakaryocyte maturation, detectable in cells at five or six days after initiation of plasma clot cultures. However, our studies utilize for the first time, in soft agar cultures, antibodies to platelet membrane glycoproteins and other markers for identification of murine megakaryocytes at an early maturation point. The specificity of these markers is further confirmed by the presence of lysozyme in murine granulocytes and macrophages, but not in megakaryocytes. From our studies, we conclude that the presence of these various antigens in megakaryocytes, derived from colony-forming cells in vitro, is another indication that megakaryocytes and their precursors synthesize von Willebrand factor, fibrinogen, and membrane glycoproteins IIIa, IIb, and GMP-140.

Recently, it has been shown that the populations of megakaryocytes in different types of murine megakaryocyte colonies have different ploidy distributions. The mean ploidy level of the "big cell" type was 16.8N/cell while the "heterogeneous" type had a mean ploidy level of only 6.8 N/cell; approximately half the cells in the latter type of colony contained 2N or 4N levels of DNA. Our studies suggest that cells at low ploidy levels expressed platelet membrane and alpha granule glycoproteins, since almost all cells in both colony types were reactive for these markers by seven days or earlier in culture. Previous studies have
shown that megakaryocytes with ploidy levels ranging from 8N to 128N can achieve cytoplasmic maturity. Our data are consistent with Paulus' observation that organelle formation is initiated in immature megakaryocytes and reaches a peak after completion of DNA synthesis (ie, endomitosis). It is unknown whether the pattern of protein expression may be altered by the maturation state of the megakaryocyte, as is the case, for example, in neutrophils, in which azurophil granules are formed only during the promyelocyte stage and specific granules are formed during the myelocyte stage. Studies are therefore in progress to further correlate maturation and ploidy levels of the cells of megakaryocyte colonies with the pattern of appearance of these various glycoproteins.

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